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Immunodiagnosis of active tuberculosis

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Abstract (<200 words)

Introduction: There is an unmet clinical need for improved diagnostic tests for active TB to provide high sensitivity for all cases, accelerate time to diagnosis and ensure timely and appropriate treatment. Whilst the measurement of *M.tb*-specific immune responses is widely used for detecting infection in the absence of TB symptoms (i.e. latent BT infection), there is currently no role for immunodiagnostics in active TB disease. This is primarily due to insufficient sensitivity, and an inability to discriminate between active disease and controlled, latent TB infection.
Areas covered: In this review we focus on recent developments in the use of immune-based test to provide a point of care test for the rule-in or rule-out of active TB.
Expert opinion: Recent studies have demonstrated that second generation IGRAs have potential use in the early rule-out of active TB, particularly in low burden settings. Newer technological platforms, including systems serology and flow cytometry, offer the means to measure specific *M.tb* specific immune signatures which have been shown to have a high level of accuracy for active TB. However, it is now crucial that new and promising undergo validation in clinically relevant cohorts which include the full spectrum of TB patients and differential diagnoses.

Keyword: Active Tuberculosis, Immunodiagnosis, Immune Response, Diagnostic test, Latent Tuberculosis.

Background

Tuberculosis (TB) is the leading infectious killer worldwide with 10.0 million incident cases and an estimated 1.3 million deaths annually (1). Upon exposure to *Mycobacterium tuberculosis (M.tb)* there is a range of possible outcomes: early clearance, transient infection (where the bacteria is presumably cleared by the immune system), or established TB infection with bacillary replication. In most infected individuals, a state of long term immune-control ensues after initiation of the adaptive immune response (known as latent TB infection [LTBI]), while approximately 2-5% of individuals will develop active TB disease, usually within the first 6 months to 2 years after infection (2–4). Typically, active TB affects the lungs (pulmonary TB), however any organ external to the lung can be afflicted (known as extrapulmonary TB [EPTB]). In order to reduce the global burden of TB, new tools are urgently required, including improved diagnostic tests. There are two main unmet needs for active TB diagnosis: 1) a rapid triage test which could rule-out active TB from the differential; 2) a test with high specificity for all active TB cases, to be used further along the diagnostic pathway.

Currently available diagnostic tools for active TB

Microbiological tests

The currently available diagnostic tools for TB infection can be separated into microbiological, radiological and immune-based tests (Table 1). Microbiological tests are the most widely used tests for the diagnosis of active TB, and this group comprises smear microscopy*, M.tb* culture and nucleic acid amplification tests (such as Gene Xpert® MTB/RIF assay, Cepheid, Sunnyvale, CA, United States). Smear microscopy is the go-to approach in resource-poor settings, and the most common method used is the identification of acid-fast bacilli in sputum samples using the Ziehl-Neelsen stain (5). Smear microscopy provides rapid results, but the findings can be confounded by individuals with non-tuberculous mycobacteria (NTM) infection, negatively impacting on the specificity of this approach. More importantly, smear tests lack sensitivity for all cases of active TB, providing as low as 60% for all TB cases (6). *M.tb* culture is considered the gold standard for detection of active TB, since it is highly specific and offers improved sensitivity over smear. However, specific laboratory equipment and conditions are required, and the test can be very slow, with some samples taking 3-6 weeks to generate results**.** Both Gene-Xpert and Gene Xpert Ultra tests have transformed TB diagnosis by providing results on the same day and providing information about drug sensitivity status. However, while the sensitivity of Gene-Xpert Ultra is approaching that of culture (7), none of these microbiological tests have high enough sensitivity to

detect the majority of active TB patients, particularly those with EPTB, HIV co-infection, and smear negative status, due to their paucibacillary nature (5). With these limitations, many patients with active TB are diagnosed clinically without definitive microbiological confirmation. Radiography and CT scans have an important role in supporting the diagnostic evaluation of TB, but are unable to reliably discriminate between TB and other diseases (8,9).

Current Immune-Based Tests

Immune-based diagnostic tools for TB include the Tuberculin Skin Test (TST) and the Interferon Gamma Release Assays (IGRAs), which are both recommended for the diagnosis of *M.tb* infection (10,11), but are not currently recommended for use in the diagnostic work-up of active TB. The TST measures induction of a delayed type hypersensitivity immune response to *M.tb* by in vivo exposure to a mixture of protein antigens from *M.tb* (purified protein derivative [PPD]). The TST is performed using the Mantoux technique of intradermal injection of 5 tuberculin units of reagent PPD (5,12). The TST has sub-optimal specificity for detecting *M.tb* infection due to PPD containing antigens which are also present in other mycobacteria (e.g. are cross-reactive), resulting in false-positive results in individuals with either prior immunization with the BCG vaccine (11) or exposure to NTM. In low-income high TB burden countries TST is still widely used in a clinical setting due to its relative cost-effectiveness and ease of use. In contrast, IGRAs work by detecting the production of interferon gamma (IFNγ) by *M.tb* -specific T-cells ex vivo, using cells collected from a blood sample. IGRAs stimulate blood cells with peptides from antigens localised in the area of the *M.tb* genome known as the Region of Difference 1 (RD-1), an area not present in either BCG or NTM (ESAT-6 (Rv3875) and CFP-10 (Rv3874)). The IGRAs represented a 100-year upgrade from the TST and provide improved specificity for detecting TB infection due to the use of *M.tb*-specific antigens (e.g. no cross-reactivity), and lack of requirement for two clinic visits (13). Currently available commercial IGRAs are the T-SPOT.TB (Oxford Immunotec Ltd, Oxford, UK), an ELISpot method quantifying IFNγ spot-forming cells, and the Quantiferon®TB Gold-In Tube (QFT-GIT (Qiagen, Hilden, Germany)) an ELISA-based method measuring IFNγ concentration in the serum of blood stimulated with *M.tb* antigens.

Table 1: The performance of currently available diagnostic tools for active tuberculosis. Diagnostic tools can be broadly categorised into microbiological, radiological and immune-based tests. For each test the relative speed, cost and diagnostic performance characteristics for detection of active TB has been described (Abbreviations: culture positive C+, culture negative C-, smear negative S-, smear positive S+, extra-pulmonary (EPTB), non-tuberculous mycobacteria (NTM)).

Test	Type of test	Speed of result	Cost (high/ low)	Sensitivity for active TB	Specificity for active TB	Notes	References
Smear microscopy AFB	Microbiological	Fast	Low	30-80%	97%	Lacks sensitivity in children, those with EPTB, and HIV+	(8,14)
M.tb culture	Microbiological	Slow (3-6 weeks)	Low	30-85%	100% Gold standard	Poor sensitivity. Poor diagnosis in children and HIV+	(9)
Gene Xpert® MTB/RIF assay	Microbiological	Fast	High	98.2% S+ 72.5% S-C+ 66% in suspected TB (pead.)	99.20%	Poor sensitivity in EPTB and HIV+ and children	(8,14)
X-ray/ CT scan	Radiology	Fast	Low/Med	~70%	~50-60%	Poor specificity. Cannot distinguish from OD	(8)
Tuberculin Skin Test	Immune-based	Fast	Low	62.5-79.5%	36.6-95.2%	Decreased specificity due to PPD cross-reacting with BCG-vaccinated individuals and those infected/exposed to NTM.	(Takwoingi et al, in press, 2019)
QuantiFERON Gold®TB In-Tube	Immune-based	Fast	Medium	67-71%	80-94%	Cannot exclude/rule-out active TB or discriminate between LTBI and active TB	(15)
T-SPOT.TB	Immune-based	Fast	Medium	80-85%	86-94%	Cannot exclude/rule-out active TB or discriminate between LTBI and active TB.	(15)

Current IGRAs or TST are able to detect approximately 80% of all adult TB cases (excluding those with HIV co-infection) in both high and low incidence settings (15–19). Whilst this high level of sensitivity is greater than microbiological tests, neither IGRA nor the TST is able to discriminate between active TB and LTBI, leading to poor specificity of these tests for active TB, particularly in high burden settings (Figure 1). Furthermore, the sensitivity is not sufficient to be reliably used as a triage rule-out test for all TB suspects, where somewhere between >90-100% sensitivity would be required, depending on the prevalence of TB in the target population (i.e. the pre-test probability). Because of this, the recommended use of immune-based tests has always been for detection of LTBI only, particularly in screening or contact tracing programmes (13,20). Historical studies have indicated that by combining immune-based tests, and/or improving on current assays by modifying the test protocol, it is possible to improve on either sensitivity or specificity for

active TB (16). In this review, we will summarise the recent developments (in both research and pharmaceutical industries) relating to the generation of improved immunodiagnostics for active TB specifically, primarily focusing on studies which have been performed in the past 4-5 years.

[Figure 1]

Evaluation of current immune-based tests for active TB

Despite being primarily used to detect LTBI, some clinicians do use immune-based tests as part of the diagnostic work-up of active TB. However, it is unclear which of the tests is optimal and in which setting. With this in mind, there have been several recent studies assessing the relative diagnostic performance of the two commercially available IGRA tests for active TB in different settings, and some studies showing improvements to the tests with minor modifications. A recent multi-centred prospective study has directly compared T-SPOT.TB and QFT-GIT assays for the diagnosis of active TB in China (a high incidence setting), using the same patients, showing concordance between the two tests and comparable sensitivity (~84%) (19). Another study conducted in South Korea found the sensitivity of T-SPOT.TB to be higher than QFT-GIT for detection of microbiologically confirmed TB cases (91% and 80.2% respectively), with the sensitivity of both tests declining significantly with increasing patient age (21). More recently, a multi-centred prospective cohort study of TB suspects in England conducted by our research group evaluated the use of existing and second generation IGRAs for the diagnostic evaluation of active TB (15). Within this large and clinically relevant cohort the T-SPOT.TB test provided 81.4% sensitivity for all active TB cases, and 84.9% for culture confirmed TB, significantly greater than the QFT-GIT test which provided 67.3% and 70.6% sensitivity respectively (15).This study demonstrates that in a low prevalence high income setting, the T-SPOT.TB is the superior immune-based test for detection of active TB, but neither test is sensitive enough to be useful as a rule out-test.

Updates to commercially available immune-based tests

In an effort to improve on the specificity of the TST for TB infection, whilst maintaining its low cost and ease of use, Staten Serum Institute (Copenhagen, Denmark) developed the C-TB test, a skin test using recombinant *M.tb* RD-1 antigens ESAT-6 and CFP-10 instead of the cross-reactive PPD. The test was found to improve on specificity for *M.tb* infection compared to the traditional TST test (22), is not confounded by prior BCG vaccination and is safe for use in TB patients

(23,24). While the C-TB test was found to have a similar sensitivity for active TB as the QFT-GIT (73.9 and 75.1% respectively), both tests provided lower sensitivity than the traditional PPD-containing TST (89.6% sensitivity), whilst the T-SPOT.TB was not included for the comparison (24). Therefore, it seems likely that the C-TB test will be more useful as an alternative test for LTBI, rather than for active TB.

In 2015/16 Qiagen introduced the Quantiferon-Gold Plus (QFT-Plus) an update on the QFT-GIT. This new test includes an additional antigen tube (TB2) with shorter peptides designed to stimulate both *M.tb*-specific CD8+ T cells (antigen/s unknown) and CD4+ T cells, in addition to the TB1 tube containing longer peptides from the ESAT-6 and CFP-10 proteins as included in the QFT-GIT (N.B the TB7.7 antigen was removed), which have been reported to primarily stimulate CD4+ T cells (25). Presence and frequencies of circulating *M.tb*-specific CD8+ T cells are known to be enriched in active TB compared to LTBI (26), and CD8+ T-cells producing IFNγ are known to be detected in some hard to diagnose TB groups such as HIV co-infected individuals (27) and children (28). The first study to evaluate the performance of the new QFT-Plus found the test had a sensitivity of 87.93% for all microbiologically confirmed active TB cases (including EPTB) (29) and reported improved diagnostic accuracy for active TB over QFT-GIT. However, the authors did not compare the new test to the QFT-GIT in all patients (only 73 patients had both test results), and thus the study was not powered to compare the two tests directly. Since then other studies have compared the QFT-Plus to the QFT-GIT within the same subjects, and found no difference in diagnostic accuracy for detecting active TB cases (30,31) or for latent TB/case contacts (32,33). However, interestingly QFT-Plus demonstrated higher sensitivity for HIV co-infected TB subjects to than the conventional QFT-GIT (34).

There have been no updates to the commercially available T-SPOT.TB assay from the main manufacturer. However, several studies have explored the use of additional antigens using the T-SPOT.TB/ELISpot platform, the results of which are discussed later (see ‘Alternative or additional antigens’).

The future for immunodiagnostics for active TB

Research groups who seek to improve on the current IGRA-like approach of detecting circulating *M.tb*-specific T cells have proposed and discovered several ways to improve on the existing tests. These approaches can be categorised into the following:

1) development of new and/or improvement of existing technological platforms,

2) investigation of host-immune responses to additional/different antigens,

3) measurement of responses using different analytes

4) immunoprofiling of *M.tb*-responding T-cells.

In this review we will discuss in depth each possible avenue for developing highly sensitive and specific tools in diagnosing representations of active TB building on existing conventional platforms (see Fig. 2 for a summary).

[Figure 2]

New technological platforms

The development of technologies to allow measurement of multiple immune parameters provides the technological basis for improving upon existing immunological test methods. The ELISA and ELISpot methods traditionally measure only one parameter per run, hence several ELISAs are needed to run to measure multiple analytes. Due to recent improvement of multiplex technologies (e.g. Meso Scale Discovery (MSD)*,* Luminex, ELLA) it is now possible to measure an array of analytes in one patient sample, and with a wide range of concentrations. Additionally, FluoroSpot, a modified method from the traditional ELISpot technique, is a novel technological platform which can simultaneously measure multiple (typically 2-3) cytokines from antigen-stimulated T-cells/PBMCs, such as IFNγ and IL-2 (35,36). Although the platform is limited in the number of parameters it can measure, it encompasses a simple and fast protocol without requiring extensive expertise. Molecular methods for chemokine/cytokine detection such as quantitative RT-PCR may provide a more sensitive method for measuring analytes, as well as making it easier to investigate additional analytes, since cDNA is easily re-probed. Flow cytometry is a powerful technique allowing for simultaneous measurement of multiple extracellular and intracellular antigens and cytokines at the single cell level. Flow cytometry and molecular facilities are already established in NHS diagnostic laboratories and while it requires a level of expertise and training for machinery operation and analysis, there has been some progress in making the technique more automated, cheaper and more standardised. Although these technologies are not new, their use in a diagnostic setting has only recently become more realistic, due to improvements in standardisation and technological accuracy, as well as reductions in cost.

Alternative or additional antigens

Since the immunodominant ESAT-6 and CFP-10 antigens were incorporated into IGRAs, it has been long proposed that further combination with additional antigens, or assays incorporating different antigens could improve current diagnostic performance of these tests. Specifically, the

incorporation of additional antigens is likely to improve the sensitivity of the test, while using alternative antigens, such as those associated with latency, has been proposed as a potential way to provide improved specificity for different states of TB infection (e.g. active TB or LTBI). *M.tb* antigens can be briefly categorised into dominant, latency, activation, reactivation and starvation antigens. The recent work on the use of additional/alternative antigens in cell based immunodiagnostic tests is summarised in Table 2.

Table 2: *Antigens identified as supporting the diagnostic of specific stages of TB infection, or improving overall sensitivity for TB infection. For each antigen the diagnostic performance and population/s in which they were tested is noted.*

Author	Antigen Stimulants	Population studied	Technique Used	Findings	Ref
Whitworth et al (2019)	ESAT-6, CFP-10, Rv3615c, Rv3879c	Patients recruited prospectively when suspected of having active TB. TB cases included EPTB and culture negative TB.	ELISpot and ELISA	With second generation T-SPOT.TB tests using novel antigens, a combination of either ESAT-6, CFP-10 and Rv3615c, or CFP-10, Rv3615c and Rv3879c, gave the highest sensitivity for active TB (with 89.2% and 88% for all TB and 94% and 93.4% for culture confirmed TB, respectively).	(15)
Arroyo et al (2018)	DosR- (Rv1737c, Rv2029c, and Rv2628) Rpf- (Rv0867c and Rv2389c)	Pulmonary TB, Latent TB	Luminex	Amongst the latency antigens tested Rv2029c gave a better performance than ESAT6/CFP10, with better sensitivity but reduced specificity.	(37)
Li et al (2017)	ESAT-6, CFP-10, Rv3615c (peptide pool)	Active TB (PTB and EPTB), non-TB respiratory disease and uninfected healthy control	ELISpot	TS-Spot (pooled antigen) showed a slight increase in sensitivity of active TB than T-SPOT.TB	(18)
Liu et al (2017)	Rv0183	Active TB, non-TB respiratory disease, and uninfected control	ELISA	Rv0183 specific IL-6 response is increased in active TB than in non-TB and healthy controls.	(38)

Shaikh et al (2017)	Rv3879c, Rv3615c, Rv3878, Rv3873, Rv2654	(Children) Recruited on a suspicion of active TB	ELISpot	Addition of Rv3615c to the standard ELISpot it gave a slight increase in performance than the standard EliSpot within this cohort but with no significant difference.	(39)
Bai et al (2016)	Rv2029c, Rv2628 and Rv1813c (latency)	Active TB, non-TB respiratory disease and uninfected healthy control	ELISpot/ ELISA	Rv1813c showed a higher IgG level in active TB than in LTBI and uninfected control.	(40)
Prabhavathi et al (2015)	Rv2626c, Rv3716c	Pulmonary TB and LTBI	ELISA	IFNγ/TNFα levels against the TB specific antigen Rv2626c or Rv3716c as a 'rule in' for LTBI.	(41)

Historic and recent studies have demonstrated that the addition of further dominant RD1-associated antigens can improve on the sensitivity for active TB when incorporated into existing immunodiagnostic tests (16). In order to provide improved sensitivity for active TB, several groups have explored using additional antigens in existing test platforms. A group in China developed a cheaper version of the T-SPOT.TB assay, called the TS-SPOT, which combines ESAT-6, CFP-10 and an additional RD-1-associated antigen, Rv3615c (EspC), into one *M.tb* peptide pool antigen well, thereby reducing cost (18). The group found comparable performance for detecting a spectrum of active TB cases (including culture negative and EPTB) compared to the standard T-SPOT.TB assay, and calculated the new assay to be more cost effective (18). However, in the recently published IDEA study, Whitworth and colleagues compared existing tests (TS-TSPOT-TB and QFT-GIT), to second generation T-SPOT tests including additional *M.tb*-specific antigens, Rv3615c and Rv3879c in active TB suspected recruited in clinical practice. Two combinations of three antigens (either ESAT-6, CFP-10 and Rv3615c or CFP-10, Rv3615c, Rv3879c – an 'ESAT-6-free IGRA') were found to give the highest sensitivity for active TB (88-89.2% for all TB and 93-94% for culture confirmed TB), and a negative predictive value of 90.0% for all TB in a high-income low incidence setting (15).The performance of the second generation tests evaluated in this study demonstrate that immunodiagnostics have the potential to be clinical useful in ruling out TB in routine clinical practice. In summary, of the RD-1-associated antigens, Rv3615c (EspC) has shown particular promise for increasing test sensitivity for active TB in recent studies (15,42).

Stimulation with DosR-regulon-encoded antigens, often termed 'latency antigens', such as Rv1733c, Rv0081, Rv2029c, Rv2628c, Rv2627 has been proposed as an approach discriminating

between active TB and LTBI, and/or for risk stratification of LTBI cases, in order to distinguish between recent and remotely infected LTBI (43,44). However, studies demonstrating potential clinical utility in appropriate cohorts have been limited. Additional, non-DosR antigens (e.g. Rv3407, Rv2660c) have been associated with immune responses in latency, and since IFNγ production to these antigens is diminished in active TB, they have the potential to discriminate between active and latent TB (45,46). Most recent work has focused on heparin-binding hemagglutinin (HBHA), a *M.tb* antigen which induces strong T-cell responses in the latent population but little/no responses in those with active TB (47), including in children (48). Recently groups have characterised the cells responsible for these LTBI-related responses to HBHA, and demonstrated that measurement of HBHA-specific IFNγ+IL2+IL17+CD4 T-cells was a strong discriminator between active and latent tuberculosis patient population (49,50). Furthermore, the protein can now be more easily produced in its methylated, immunogenic form using *M. smegmatis*, increasing the likelihood of scale-up and future development of these tests (18,51). A recent systematic review of the use of novel *M.tb* antigens revealed that latency associated antigens (DosR and non-DosR encoded) were the most used novel antigens by groups seeking to improve test performance, and despite numerous studies demonstrating their immunogenicity in multiple studies, evidence for performance in clinically relevant cohorts is limited (52).

New analytes or combinations of analytes

Recent studies have been increasingly interested in measuring host responses alternative to, or downstream of IFNγ. By measuring additional cytokines and chemokines produce in response to *M.tb* antigens, tests can be more sensitive, and may additionally provide discrimination between active TB and LTBI.

Host biomarker studies have largely placed CXCL10 (IP-10) in the centre of interest as a potential alternative to IFNγ. CXCL10 is produced in large amounts by monocytes, neutrophils and other cells in response to IFNγ signaling, thereby amplifying the TH1 CD4+ T cell IFNγ response to antigenic stimulation. The amplificatory effect of IFNγ on CXCL10 is believed to be why it has demonstrated the greatest promise in improving the sensitivity of IGRAs in studies. Studies have shown that there is significantly elevated plasma levels of CXCL10 in active TB patients after stimulation (53,54) as well as a greater sensitivity (90.9% vs 95.5%) when discriminating between active TB and LTBI, compared to using IFNγ alone (53). Further studies have shown greater diagnostic ability of CXCL10 over IFNγ when differentiating TB from other respiratory diseases (55), for detection of infection in household contacts (56) as well as picking up patients who have

recently converted TST status (57). However, not all studies have shown CXCL10 to perform better than IFNγ (58). Overall, CXCL10 has shown great potential as a replacement to IFNγ (see Table 3), but larger studies using prospective cohorts of active TB suspects are required to fully ascertain whether new tests should adopt this marker as the primary read-out.

Table 3 *Selected studies investigating the use of CXCL10 in the diagnosis of TB, detailing the diagnostic sensitivity of CXCL10 for the diagnosis of Active TB (ATB) compared to IFNγ alone in these studies. ATB= Active TB, LTBI= Latent TB Infection, OD= Other Pulmonary Diseases, HC= Healthy Controls, HHC= Healthy Household Contacts, QFT= Quantiferon, QFT-GIT: Quantiferon Gold.*

Study. Author (Year)	Location	Cohort	Stimulation/ Test	Results: does CXCL10 improve sensitivity for the diagnosis of ATB compared to IFNγ?	**Ref**
Nonghanpithak et al (2017)	Thailand	48 APTB, 200 HC with TB contact history, 52 HC	QFT	Combining IFNγ with CXCL10 provided 97.9% sensitivity which is significantly higher than IFNγ alone ($p < 0.001$)	(57)
Tebruegge et al (2015)	Australia	6 ATB, 16 LTBI, 75 Uninfected, 28 "Common Discordance"	ESAT-6 (CFP-10, PPD)	CXCL10 alone had a greater sensitivity of 95.5% compared to 90.9% in IFNγ	(54)
Jeong et al (2015)	Korea	30 ATB, 44 LTBI, 25 controls	QFT-GIT	93.9% sensitivity when discriminating ATB out of the TB-related subjects using the mitogen: antigen-specific ratio. This was greater than IFNγ.	(59)
Latorre et al (2014)	Spain	Paediatric cohort: 12 ATB, 81 Contact-tracing patients; 137 known LTBI	QFT-GIT	CXCL10 sensitivity was lower than IFNγ (66.7% and 91.7% respectively). Combining the analytes showed no further improvement.	(60)

In addition to CXCL10, other CC chemokines other are known to play an important part in the immunological response to *M.tb*, attracting monocytes and T cells as well as assisting in the formation of granulomas. Earlier research simply measured unstimulated serum levels, however more recent studies have adopted an antigen-specific approach. Following ESAT-6/CFP-10 stimulation, MCP-1 and MCP-3 are found to be higher in active TB (61–63) but they must often be measured in combination with additional markers for any meaningful diagnostic accuracy, such as with MIP-1β (63–65) or CXCL10 (66). Perhaps the most promising chemokine biomarker to come out of the past few years has been CXCL9 (MIG). Like CXCL10, CXCL9 is produced by cells in response to IFNγ, and is specifically released in response to stimulation with *M.tb* antigens (67).It is known to be produced in higher levels by active TB patients (68), and provides greater diagnostic performance than IGRAs when used in combination with other analytes such as IL-8 or I-TAC (69).

Th1 cytokines other than IFNγ, such as TNFα and IL-2, are known to be expressed by *M.tb* specific T-cells in active disease (70) and play a crucial role in protection against *M.tb* (71) and are therefore obvious alternative targets for immunodiagnostic tests. Antigen-specific studies show higher levels of TNFα produced by pulmonary TB patients and TB pleurisy patients but that measurement of TNFα alone have a lower diagnostic sensitivity than IFNγ (57). However, TNFα has been shown to improve diagnostic accuracy when used in combination with other cytokines and chemokines to detect active TB (57). Most recently, a study developed a TNFα Release Assay (TARA) which improved specificity for detecting active TB when used in combination with the traditional IGRA (72). On its own, IL-2 has shown promise as a discriminator between active TB and LTBI in some studies (21,62,73)and a useful tool for detecting active TB in both children (74) and adults (55,64,75,76).

Several groups have explored the use of multiple cytokines to improve diagnostic sensitivity of IGRAs. Several groups have demonstrated that combining IFNγ with CXCL10 and IL-2 can increase the detection accuracy for active TB (57,77). When detection of IL-2 and CXCL10 were combined with QFT-GIT, the sensitivity for active TB rose to 95.5% which was a statistically significant increase compared to QFT-GIT alone (78). However, a 2017 study using PPD for antigen stimulation on PBMCs found that supplementing the T-SPOT IFNγ result with IL-2 and IL-10 provided a lower sensitivity for detecting active TB compared to the T-SPOT.TB alone (79). Thus, the evidence points towards additional cytokines and chemokines featuring primarily as supplementary biomarkers to IFNγ in order to maximise diagnostic potential in most studies, but

these findings warrant validation in prospective studies of unselected patients with suspected TB in routine practice.

A recent study by Chegou et al. explored the diagnostic performance of an array of target cytokines and chemokines using the QFT-GIT platform and a customised Luminex technology for detection, for the detection of PTB (65). They found that the specificity for active TB in both HIV infected and uninfected adults could be optimised by combining unstimulated levels of IFNγ, MIP-1β and TGF-α with stimulated levels of TGF-α and VEGF (65), findings which they validated in an independent cohort comprising multiple study sites. The integration of unstimulated and stimulated measurements is a particularly novel approach to identify improved diagnostic algorithms using these tests. However, the diagnostic performance provided was too poor to be clinically useful (68.9% sensitivity and 83.1% specificity in the training set, and 64.2% and 82.7% in the test set, respectively), and the use of such a complex algorithm may introduce issues of poor reproducibility and standardisation, and furthermore, may not have provide comparable diagnostic performance in other settings where baseline cytokine and chemokine profiles are likely to differ. Other studies have identified further analytes such as IL-4 (80), IL-8 (81), IL-9 (82), IL-13, IL-15, IL-16 (62,68) and MMP-9 (65)which have shown some promise as auxiliary biomarkers.

Phenotype and function of *M.tb* specific T cells

To improve on the specificity of immunodiagnostics for active TB several groups have explored the *M.tb*-specific T-cell response with greater complexity by using flow cytometry to simultaneously measure phenotypic and functional markers of *M.tb*-specific T cells. Multiple studies have now demonstrated that cellular immune signatures measured in this way have the potential to be used as immune-based tests to allow for discrimination between active and latent TB (Table 4). Previous studies evaluating the role of functional T-cells as biomarkers for active TB, such as the proportion of *M.tb*-specific CD4+ TNFα-only cells (e.g. IFNγ-IL-2-TNFα+) which was found to discriminate between active TB and LTBI (83,84), have not shown consistent results in subsequent (85). Hence more recent studies have focused primarily on the activation and/or memory differentiation state of cytokine-producing T-cells, which has in recent years shown promising and reproducible results across research groups.

Despite the high diagnostic performance of the differentiated effector memory (i.e. $CD45RA^-$ $CCR7^-CD127^-$) phenotype signature (T_{EFF}) within the CD4 TNFα-only population which performed

with very high diagnostic accuracy for detection of active TB from remote LTBI in two studies from our lab (86,87). The focus by other groups has been on the CD4+ IFNγ-producing *M.tb*-specific cells. Several groups have reported using the memory/differentiation marker CD27 within this population to differentiate active TB from LTBI patients (25,48,84,85, 86). Different approaches to incorporating this marker into a cellular immune signature have been taken: i.e. by measuring the levels of CD27 as a ratio in relation to the parent population (also known as the TAM-TB assay (88)), measuring the presence/absence of expression in combination with the memory marker CD45RA (90), or by combining CD27 marker expression with expression of both IFNγ and TNFα (91). The discrimination between active TB and LTBI was high in these studies particularly for the %CD27-CD45RA- signature (90). More recently, the incorporation of activation markers in cellular immune signatures, first investigated by Adekambi et al., has been shown to provide accurate discrimination between LTBI and active TB (92), and in the case of the HLA-DR antigen, this high performance has been validated by independent groups, and including in patients with/without co-infection with HIV (85,93). These encouraging studies suggest that T cell signatures identified using flow cytometry could act as highly specific tests for active TB in immune responses individuals, and could perhaps therefore functional as a rapid second line test after obtaining a positive IGRA.

Flow cytometry is already established in many NHS diagnostic laboratories throughout the UK and other high income countries and is suitable for routine diagnostic use (94). These signatures therefore warrant prospective validation in a large-scale independent cohort in routine clinical practice.

Table 4: The discriminatory ability of cellular immune signatures between active tuberculosis and latent tuberculosis infection according to recent literature. The *M.tb*-specific CD4+ and CD8+ T-cells have been the focus of investigators, who have explored differences in the functional, memory phenotype and activation profiles of these cells. Some of the signatures have been validated in independent studies, as indicated.

Original study	**Study design**	**Type of immune signature**	**Sensitivity for active TB**	**Specificity for active TB**	**Validated**	**Refs**
Harari et al., (2011)	*Parent cells*: CD4+ IFNγ T-cells *Stimulation:* ESAT-6/CFP-10 *Signature Markers:* IFNγ, IL-2, TNFα *Signature:* % TNFα-only	Functional	100% / 66.7%	96% / 92.4%	No	(83,95)
Pollock et al.(2013)	*Parent cells*: CD4+ TNFα-only cells *Stimulation:* ESAT-6/CFP-10 & PPD	Memory Phenotype	100%	92.9%	Yes	(86,87)

	Signature Markers: IFNγ, IL-2, TNFα, CD45RA, CCR7, CD127 *Signature:* % CD45RA-CCR7-CD127-					
Rozot et al.(2014)	*Parent cells:* CD4 T-cells and CD8+ T-cells *Stimulation:* ESAT-6/CFP-10 *Signature Markers:* IFNγ, IL2, TNFα *Signature:* % TNFα-only (of CD4+ T cells) and % IFNγ (of CD8+ T cells)	Functional	81.1%	86.5%	No	(26)
Portevin et al.(2014)	*Parent cells*: CD4+ IFNγ+ T-cells and CD4+ T cells *Stimulation:* ESAT6/CFP10 & PPD *Signature Markers:* IFNγ, CD27 *Signature:* Ratio of MFI of CD27	Memory Phenotype	83.3% / 83.3%	83.7% / 96.8%	Yes	(88,89)
Petruccioli et al (2015)	*Parent Cells:* CD4+ IFNγ T-cells *Stimulation:* ESAT6/CFP10 *Signature Markers:* CD45RA, CD27 *Signature*: % $CD45RA^{-}CD27^{-/+}$	Memory Phenotype	61.54%-76.92%	86.67%-91.67%	No	(90)
Adekambi et al (2015)	*Parent Cells*: CD4+ IFNγ+ T-cells *Stimulation:* ESAT-6/CFP-10, *Mtb* cell wall antigens & PPD *Signature Markers:* HLA-DR, CD38, Ki-67 *Signature:* $\%HLA\text{-}DR^{+}$ or $\%CD38^{+}$ or $\%Ki\text{-}67^{+}$	Activation	100% / 100%	96% / 95%	Yes (for %HLA-DR+)	(85,92,95)
Hutchinson et al (2017)	*Parent Cells*: a) CD4+ $GM\text{-}CSF^{+}$ b) CD4+ T cells *Stimulation:* ESAT6/CFP10 & PPD *Signature Markers:* *Signatures:* a) $\%CD27^{-}$ b) %CD154+ $TNF\text{-}\alpha^{+}$ CXCR3+	Memory Phenotype	Not reported AUC (a)=0.9277 (b)=0.855	Not reported AUC (a)=0.9277 (b)=0.855	No	(50)

B cell / antibody tests for active TB

Serological tests to measure antibodies to immundominant antigens have several advantages over cellular based assays, including speed, reduced cost and ease of use in the field. However, it has been historically believed that antibodies play little or no role in the immune protection to TB infection/disease in humans (96) and that diagnostic tests incorporating *M.tb*-specific antibodies lacked sufficient test accuracy (97). Indeed, when measured using crude approaches, there appears to be considerably variable sensitivity and specificity for active TB between studies, and despite widespread use in some countries (especially India), their use is not recommended by international guidelines for TB diagnosis such as WHO (97).Some recent studies have challenged this, and suggested that antibodies, and B cell responses more generally, may well play an important role in protection against TB infection/disease and relate to different states of TB infection (18,98–100). In a ground breaking paper that demonstrated that when the profile and functionality of *M.tb*-specific antibodies are examined in more detail in an unbiased systems

serology approach, Lu et al demonstrated that distinct antibody profiles are observed between active TB and LTBI individuals (98). Specifically, *M.tb*-specific antibodies produced by LTBI individuals were functionally superior, leading to improved killing of the bacteria in in vitro using cytotoxicity assays, and possess different glycosylation profiles (98).

Recently, Joosten et al demonstrated that B cells displayed impaired functionality in active TB and LTBI, which improves upon treatment. Using a growth inhibition assays, the group described that B cells play a role in T-cell priming and effector function (101). These new studies identifying specific roles for antibodies and B cells in TB disease and indicating potential discriminatory ability between TB states is leading to speculation that detection of these responses could be exploited for new immunodiagnostic tests development. In parallel, some groups have recently identified immunodominant antigens such as Rv0310c and Rv1255c, which, when used to measure *M.tb*-specific immunoglobulins, can improve on the sensitivity of traditional used antigens such as Ag85, ESAT-6 and CFP-10 (102). However, another multi-centred study tested the diagnostic performance of immunoglobulins specific to 57 *M.tb* antigens, singularly or in combination, for detection of active TB patients, but found poor specificity when assessing for high sensitivity, as well as variability in antigen responsiveness between different locations (103). In summary, it appears that new approaches incorporating the measurement of phenotype and function of *M.tb*-specific antibodies will be necessary to provide improved the diagnostic performance of these types of tests.

Expert Commentary

In order to fully address the global TB burden, we sorely need improved diagnostic tests for active TB. These new tests should seek to address the unmet needs for this patient group, i.e. a rapid triage rule-out test which can be used in the early stages of diagnostic work-up, as well as a highly specific test which can be user later in the diagnostic pathways, to provide an accurate rule-in of active TB cases which are currently missed by standard tests (paucibacillary TB and extra-pulmonary TB in particular). In recent years, there has been significant progress towards the development and evaluation of new immunodiagnostics to meet both of these unmet needs for active TB diagnosis. A range of approaches has been taken to either improve on existing platforms (e.g. by using of additional or alternative antigens, detection of additional/alternative markers), or use different technologies to allow detection of alternative immune signatures/biomarkers which can provide improved accuracy for active TB. The use of additional

and alternative *M.tb*-specific antigens has led to improved second generation IGRA tests. A prospective cohort study evaluating existing and second generation IGRAs has demonstrated that the use of 3 *M.tb* antigens (either ESAT-6, CFP-10 and Rv3615c, or CFP-10, Rv3615c and Rv3879c) combined in the T-SPOT.TB platform can provide ~90% sensitivity and a high negative predictive value for all active TB cases, in a cohort reflecting routine clinical practice (Whitworth, 2019). Thus, despite their low specificity for active TB (due to detection of LTBI cases), this study shows that IGRA-based platforms can be modified to provide improved sensitivity, to a level at which they can be clinically useful as a triage rule-out test, early in the diagnostic work-up of active TB suspects. A widely investigated strategy to improve current immunodiagnostic platforms is by incorporating for measurement of additional or alternative analytes, such as CXCL10. However, the studies have yet to demonstrate consistent improvement in diagnostic performance, and/or utility in a prospective and clinically relevant cohort of TB suspects.

With regards to the need for rapid tests with improved specificity for all active TB cases, progress has been made in the discovery and evaluation of cellular immune signatures, measuring the phenotype of *M.tb*-specific cytokine producing T cells using flow cytometry, some of which have consistently provided a high level of accuracy for active TB cases in independent studies (63,85,87). Such tests may provide the ability to both identify active TB in those where a detectible *M.tb*-specific immune response is identified, and also discriminate between active TB and LTBI, allowing them to serve as an accurate rule-in test for TB suspects. Such a test could be utilised either to allow acceleration of a positive culture result, or to provide an accurate rule-in of active TB when other tests have either proved not possible (i.e. sample site inaccessible for microbiology), or else provided false negative results (e.g. smear).

Progress has also been made in the field of antibody-based diagnostic tests for active TB, specifically by measuring the phenotype and functional profile of *M.tb*-specific antibodies. However, further work is required to develop this methodology, and to determine whether such approaches have the ability to provide an improvement over current diagnostic tests.
Future work on these novel approaches must focus towards the evaluation of new tests in clinically relevant cohorts, incorporating the full spectrum of TB patients and differential diagnoses

Five year view

In this review we have summarised the last 4-5 years of progress on development of the improved immunodiagnosis of active TB, during which time some promising research has indicated that

improved tests may well be on the horizon. Technological progress towards measurement of multiple analytes, using smaller amounts of sample, will no doubt eventually lead to cheaper, quicker and more accurate diagnostic tests. However, with the exception to studies assessing both existing or second generation IGRA and TST based tests, and the chemokine/cytokine signatures measured by Chegou et al, so far very few of the novel promising assays have been validated in a prospective cohort recruited during routine clinical practice (65). This validation step is crucial to fully evaluate the potential clinical utility of these new approaches to the diagnosis of active TB. It is important to note that while improved sensitivity of immune-based assays for active TB may well be a possibility, it is unlikely that the traditional IGRA or TST type set up, where an incubation period is required before the measurement of the adaptive immune response (whether in vitro IFNγ production or in delayed type hypersensitivity reaction), would meet the low cost and high speed requirements for a rapid rule out test, as laid out in the target product profile for new TB tests outlined by the WHO (1). The measurement of antibody sub-types, however, could possibly meet such requirements if found to be sufficiently sensitive for the full spectrum of active TB cases.

Key issues

- Current commercial IGRAs do not have high enough sensitivity to act as a rule-out test for active TB or even have high enough specificity to discriminate between the different stages of infection and disease.
- Second generation IGRAs, incorporating additional *M.tb* antigens, have the potential to be used as a triage rule-out test for active TB suspects
- Though many new tests have shown improvement in sensitivity and specify in a case control cohort setting, promising assays must be validated in a well-defined clinical cohort which includes all the hard to diagnose TB cases (i.e. EPTB and paucibacillary TB).
- The findings of studies evaluating diagnostic performance from a low endemic setting are unlikely to be generalizable to high endemic settings, given the varying rates of infection and the differing manifestations of TB disease.
- Furthermore, many of the immunodiagnostic test platforms are too costly and/or complex to be used in many rural or low-income country settings.
- Future research in the field of immunodiagnosis for active TB should focus towards a point-of-care test.

- Ultimately, we must ensure that promising new tests are appropriately validated in large prospective cohort studies where participants are enrolled during routine clinical practice.

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Annotations: * = of interest, ** = of considerable interest

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Declaration of interest
AH reports a patent application pending relating to the use of T-Cell Diagnosis in Tuberculosis infection. AL is a named inventor on patents (EP05729257.5, EP1735623[B1], US8,105,797[B2], EP2069792, P2069792[B1], EP2005182, EP2005182[B1], US8,765,336[B2], EP10716313.1, EP2417456[B1], US9,377,460[B2], US9360480[B2], EP0941478[B2], EP1152012[B1], P1735623[B1], US8105797[B2], EP1144447[B1], and US9005902[B2]) pertaining to T-cell-based diagnosis, including current and second-generation IGRA technologies. Some of these patents were assigned by the University of Oxford, Oxford, UK, to Oxford Immunotec plc, resulting in royalty entitlements for the University of Oxford and AL.

Figure headings

Figure 1: The performance of currently available diagnostic tests for detection of active TB compared to the ideal. The central box represents all active TB cases, which includes a spectrum of manifestations from mild to severe (left to right). The coloured circles/ovals show the relative sensitivity and specificity of the currently available tests for active TB. Microbiological Culture (purple), and Gene Xpert (pink) tests are highly specific for active TB, but lack sensitivity for the cases with lower bacterial load and/or extra-pulmonary TB. Smear microscopy (light blue) also lacks sensitivity for these cases, and has compromised specificity sue to detection of other acid fast bacilli. Immune-based tests such as IGRAs (green) have the greatest sensitivity for all active TB cases, but lack specificity due to detection of latent TB infection (LTBI). In this review we focus on the areas of research which may lead to improvement on immune based tests for TB, either by improving sensitivity, or specificity. Ideally, a new immunodiagnostic test (green dashed line) would detect the full range of manifestations of active TB, and not result in any false positives. The images of the infected lung sites in this figure are reprinted from The Lancet, Vol 387. Keertan Dheda, Clifton E Barry, Gary Maartens, Tuberculosis, Pages No. 1211-1226, (104) Copyright (2016), with permission from Elsevier.

Figure 2: Strategies to improve IGRA tests for the diagnosis of active TB. IGRAs work by detecting the production of IFNγ by *M.tb*-specific T-cells. The incorporation of new technological platforms, the measurement of alternative/additional analytes and the use of alternative/additional

M.tb antigens, are the main approaches that have been proposed to advance the performance of IGRA tests in diagnosing of active TB.